Observations on the estimation of amino groups in silk using ninhydrin reaction

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The hypothesis that the reaction between the ninhydrin and the amino groups of protein is quantitative, 2 mol of ninhydrin quantitatively react with 1 mol of the amino acid, and a certain minimum concentration of ninhydrin is required for a given weight of protein if the reaction were to complete has been confirmed.

Keywords: Ninhydrin, Ruhmann’s purple. Silk, Triketo hydridene hydrate

1 Introduction

Various methods such as potentiometric titration and 1-fluoro-2, 4-dinitrobenzene (FDNB) technique have been reported for the determination of amino groups in proteins and polyamides. The use of ninhydrin (triketo hydridene hydrate) for the quantitative estimation of amino groups in proteins is another popular technique. Ninhydrin, a powerful oxidizing agent, causes the oxidative deamination of amino groups, liberating ammonia, carbon dioxide, the corresponding aldehyde and a reduced form of ninhydrin. The liberated ammonia then reacts with an additional mol of ninhydrin and reduced ninhydrin to yield a purple substance known as Ruhmann’s purple (Scheme 1) which absorbs maximally at 570 nm (Fig. 1). Because this absorption is nearly a linear function of the amount of amino groups originally present, the ninhydrin reaction provides a convenient and quantitative colorimetric assay for amino groups.

Application of ninhydrin reaction for the determination of amino groups in textile fibres such as wool, nylon 6, nylon 6.6 and silk has been attempted by many researchers. Knot and Rossbach used this technique for the determination of amino groups in nylon 6 and nylon 6.6 and found that ninhydrin method shows good agreement with the other methods and has good reproducibility. They suggested a reaction time of 30 min in a boiling water bath, an external standard of amino caproic acid solution and a sample weight of 20 mg.

In another attempt to determine the α + ε amino groups in wool, Knot et al. studied the influence of wool fibre diameter and cystine content on the determination of amino groups and suggested that a sample weight of 10 mg and a Leucine external standard solution should be used for wool. From their studies on wool, nylon 6 and nylon 6.6, Knot et al. found that this method is also applicable to the dyed materials.

Shimizu et al. determined the amino groups in silk and suggested a reaction time of 3 h, an external standard solution and a sample weight of 20 mg. They also studied the rate of glycine-ninhydrin reaction.

Scheme 1—Reaction between ninhydrin and amino acid

Fig. 1—Absorbance spectra of Ruhmann’s purple developed by glycine-ninhydrin reaction
hydrin and silk-ninhydrin reactions and the storage stability of the ninhydrin solution.

Friedman and William studied the reaction of ninhydrin with several insoluble fibrous proteins such as wool, mohair, human hair, sheep horn, feather and silk to determine the conditions for optimum production of coloured ninhydrin derivative (Ruhmann's purple). They suggested the use of 40-60% dimethyl sulphoxide as solvent and measured the percentage of purple colour yield, contributed by ε-NH₂ groups, for all these proteins.

Wang and Pailthorpe used the ninhydrin method to measure the extent of hydrolysis of peptide linkages in wool fibre. Milligan and Wolform applied this technique to measure the extent of modification of amino groups in wool on acetylation. Quantitative analysis of the amino groups in the acetylated silk using ninhydrin has been reported by Mearl et al. In a study correlating the fixation of acid dyes with amino groups and the thermodynamics of acid dyeing of silk, Ritu De Giorgi used this technique to determine the amino groups, as suggested by Knot et al. for wool, but used a reaction time of 45 min and a sample weight of 20 mg. The need to use more quantity of silk (20 mg) compared to wool (10 mg) has also been stressed by these workers as the amount of primary amino groups is lower in silk fibroin than in wool. Knot et al. have shown that this method can be used as a tool to assess the degree of degumming of silk.

It is worth pointing out that the majority of the researchers used 2 ml ninhydrin solution (2% conc. in suitable solvents) and a temperature of 98-100°C. However, variations in terms of weight of silk sample and time of treatment have been observed.

The present paper is based on the thinking that the reaction between ninhydrin and amino groups of protein is quantitative and according to the reaction, 2 mol of ninhydrin quantitatively react with 1 mol of amino acid. Therefore, a certain minimum concentration of ninhydrin will be required for a given weight of protein if the reaction has to complete. Such a hypothesis was based on the observation that the amino group estimation, obtained by single treatment of a known weight of silk with a known volume of ninhydrin, did not tally with the total amino groups obtained by the repeated treatment of silk with ninhydrin.

2 Materials and Methods

Degummed and bleached 100% mulberry silk (Bombyx mori) weighing 48 g/m² was used.

Ninhydrin (AR), pyridine (LR), isopropanol (LR), sodium propionate (LR), methyl cellosolve (LR), propionic acid (LR) and glycine were used.

2.1 Preparation of Ninhydrin Solution

20.18 g sodium propionate and 2 g ninhydrin were dissolved in 50 ml of methyl cellosolve and 9.3 ml of propionic acid and the solution was made to 100 ml with distilled water. The ninhydrin solution so prepared has the storage stability of about 20 days in a refrigerator after which the yellow colour solution turns to black-brown.

2.2 Preparation of Glycine Standard Solution

As the N-terminal groups are contributed mainly by glycine, alanine and serine in silk, glycine was taken as the external standard solution. 2 x 10⁻³ M of glycine solution was prepared in isopropanol:water (10:90) mixture.

2.3 Silk-Ninhydrin Reaction

2 ml of ninhydrin solution and 1 ml of pyridine:water (10:90) were taken in a test tube. 50 mg of silk fabric was introduced to it. The test tube was then capped with an aluminium foil and placed in a boiling water bath for 35 min. The water level in the bath was maintained constant by replacing the water evaporated from the bath. After the reaction time (35 min) was over, the test tube was allowed to cool, uncapped and the solution was transferred to a 50 ml volumetric flask. The optical density of this solution was measured at 570 nm with reference to a blank solution prepared without silk and subjected to similar treatment as mentioned above.

2.4 Glycine-Ninhydrin Reaction

The reaction procedure mentioned in 2.3 was followed except that 0.2, 0.4, 0.6, 0.8 and 1 ml of glycine were taken in place of silk. The optical density of the purple solution obtained was measured at 570 nm with reference to a blank solution prepared without silk and glycine.

From the concentration of glycine and the optical density of purple solution, the glycine standard calibration curve was obtained (Fig. 2).

2.5 Determination of Amino Groups in Silk

The amino groups in silk were determined by different methods.
In the first method, the optical density of the purple colour solution obtained in silk-ninhydrin reaction was compared with the glycine standard curve, and the amino groups content was calculated as follows:

\[
\text{Amino groups (eq/g)} = \frac{x \times 50}{1000}
\]

where \( x \) is the concentration of glycine in mol/l from the standard curve corresponding to the optical density of the purple colour developed by silk-ninhydrin reaction.

The second method avoids the reference to standard curve. In this method, three test tubes, one with silk and ninhydrin solution, other with glycine standard solution (1 ml) and ninhydrin solution, and the third one (blank) without glycine and silk were run parallel. After making each solution to 50 ml, the optical densities of the first two purple solutions were measured with reference to that of the third one. The amino groups content was determined as follows:

\[
\text{Amino groups (\mu mol/g)} = \frac{\text{Quantity of glycine in 1 ml standard solution (\mu mol)}}{\text{Mass of the silk (g)}} \times \frac{\text{Optical density of silk}}{\text{Optical density of standard solution}}
\]

3 Results and Discussion

In the determination of amino groups in silk using the ninhydrin reaction, different workers have used the reaction temperature at or near boil, but different quantities of silk (10-20 mg) and reaction periods (30, 45, 60 and 180 min). We thought of standardizing the variables such as the weight of silk, the quantity of ninhydrin solution, and the time and temperature of reaction. The effects of various parameters are discussed below.

3.1 Effect of Reaction Time and Temperature on Purple Colour Development

The glycine-ninhydrin and silk-ninhydrin reactions were carried out at 80, 90 and 98°C for 10, 20, 30, ..., 110 min. After the reaction time was over, the solutions were made to 50 ml with distilled water and the optical densities were measured at 570 nm using the corresponding blank solution in the reference cell. The quantities of silk and glycine solutions used were 50 mg and 1 ml (2 x 10^3 mol/l) respectively.

Fig. 3 shows that the rate of reaction increases with the increase in temperature. The maximum development of purple colour takes place in 35 min at 98°C, in 50 min at 90°C and in 80 min at 80°C. After the peak, a continuous drop is observed in the optical density at 570 nm. It is also observed that after attaining the peak, the purple colour changes to pink which has also been confirmed from the change in \( \lambda_{\text{max}} \). The trend is similar for silk and glycine.

3.2 Effect of Reaction Time and Temperature on Absorbance of Blank Solution

The blank solution when subjected to conditions similar to silk-ninhydrin reaction, shows a continuous increase in the colour value with time. Here also, an increase in the temperature increases the rate of colour change (Fig. 4).

Optical density measurements were made using distilled water as the reference at 570 nm though the \( \lambda_{\text{max}} \) for the blank solution was not 570 nm.
3.3 Effect of Storage Time on Degradation of Purple Colour

Based on the optical density measurements, it has been found that the intensity of the purple colour developed due to the silk-ninhydrin and glycine-ninhydrin reactions decreases rapidly with time. The degradation of purple colour on storage is shown in Table 1. It is clear from the table that with the increase in storage time, the degradation increases and in 48 h the purple colour totally disappears. The rate of degradation of purple colour developed by silk-ninhydrin reaction is slightly faster. The colour strength of the blank solution also decreases with time.

3.4 Effect of Reaction Time on the Determination of Amino Groups in Silk

Table 2 shows that the amino groups involved in the reaction with ninhydrin increased continuously with the increase in reaction time up to 35 min. However, as the purple colour starts changing to pink, the optical density at 570 nm shows a decreasing trend thereafter. Therefore, 35 min has been taken as the standard time required for the reaction to be completed at 98°C.

3.5 Successive Treatments of Silk with Ninhydrin

In the reaction of silk with ninhydrin, the fibrous form of the sample is retained, and since the reaction is the quantitative one, it was thought that this method can be used for the deamination of silk. Keeping this in mind, we estimated the residual amino groups in the silk subjected to ninhydrin test and found that the removal of amino groups from the silk is not complete in a single treatment with ninhydrin. The residual amino groups in silk in successive treatments are shown in Fig. 5. The true estimation of the amino group content can be done only on summation of the amino groups estimated by the successive ninhydrin test as shown in Table 3.

Alternatively, we thought that the reaction between silk and ninhydrin under the given condition of time, temperature, weight of silk and volume of ninhydrin did not go to completion, leaving residual amino groups in silk. Our standardization of parameters suggested that the chosen time and temperature (35 min, 98°C) are adequate for the reaction to complete. However, we were not very sure about the adequacy of the quantity of ninhy-
Table 3—Effect of adequate and inadequate quantity of ninhydrin on the determination of amino groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amino groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>With inadequate quantity of ninhydrin (successive treatments)</td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>3.68</td>
</tr>
<tr>
<td>Second</td>
<td>1.65</td>
</tr>
<tr>
<td>Third</td>
<td>1.14</td>
</tr>
<tr>
<td>Fourth</td>
<td>1.03</td>
</tr>
<tr>
<td>Fifth</td>
<td>0.81</td>
</tr>
<tr>
<td>Sixth</td>
<td>0.42</td>
</tr>
<tr>
<td>Total</td>
<td>8.73</td>
</tr>
<tr>
<td>With adequate quantity of ninhydrin</td>
<td>9.08</td>
</tr>
<tr>
<td>First</td>
<td>negligible</td>
</tr>
<tr>
<td>Second</td>
<td></td>
</tr>
</tbody>
</table>

Table 4—Effect of ninhydrin concentration on the estimation of amino groups in silk

<table>
<thead>
<tr>
<th>Ninhydrin concentration ml</th>
<th>Amino groups ( \times 10^{-5} ) eq/g Based on the optical density measured at 570 nm</th>
<th>Based on the optical density measured at corresponding ( \lambda_{max} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.68</td>
<td>3.68</td>
</tr>
<tr>
<td>4</td>
<td>3.74</td>
<td>3.82</td>
</tr>
<tr>
<td>6</td>
<td>4.62</td>
<td>4.76</td>
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<tr>
<td>8</td>
<td>5.59</td>
<td>5.84</td>
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<td>10</td>
<td>6.39</td>
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<tr>
<td>18</td>
<td>9.08</td>
<td>9.64</td>
</tr>
<tr>
<td>20</td>
<td>9.08</td>
<td>9.64</td>
</tr>
</tbody>
</table>

Fig. 6—Effect of ninhydrin concentration on \( \lambda_{max} \) of silk-ninhydrin reaction product

Fig. 7—Effect of ninhydrin concentration on optical density of silk-ninhydrin reaction product

drin solution used (2 ml of 2% ninhydrin solution) for the given weight (50 mg) of silk sample. This hypothesis was confirmed by conducting following experiments.

3.5.1 Effect of the Quantity of Ninhydrin Solution on the Estimation of Amino Groups in Silk

The silk-ninhydrin reaction was carried out with 50 mg of silk and 2, 4, 6, ..., 24 ml of ninhydrin solution to see the effect of ninhydrin concentration on the determination of amino groups.

With the increase in the concentration of ninhydrin in the reaction medium there was a continuous shift in the \( \lambda_{max} \) (Fig. 6) as the colour changed to reddish hue. The optical density values corresponding to the ninhydrin concentration in the reaction medium at 570 nm and those obtained at corresponding \( \lambda_{max} \) are plotted in Fig. 7. The figure shows that the amino group content increases with the increase in ninhydrin concentration in the reaction medium. The optical densities reach a peak and then level off. The amino group content calculated from the optical density values corresponding to the concentration of ninhydrin are given in Table 4.

These experiments clearly indicate that the ninhydrin concentration cannot be arbitrarily selected for a given weight of silk or protein sample. This has been further substantiated by taking different weights of silk sample and finding out the optimum amount of ninhydrin required for the ninhydrin-silk reaction to complete. A linear relationship obtained between the ninhydrin concentration required for the reaction and the given weight of silk (Fig. 8) clearly indicates the quantitative nature of the reaction between ninhydrin and protein. For this reason, in our earlier experiments when 50 mg of silk was treated with 2 ml of ninhydrin solution, the residual amino groups in silk were obtained during the successive treatments of silk with ninhydrin solution.

If the ninhydrin concentration is adequate enough in the reaction medium, then it should be possible to eliminate all the amino groups from
the silk in a single treatment. Alternatively, in the absence of sufficient concentration of ninhydrin, the protein must be subjected successively to ninhydrin test in order to get the true picture of the total amino group content of the protein. We have confirmed both these aspects. When the ninhydrin concentration was inadequate in the reaction medium, residual amino groups were present in silk which could be eliminated with successive treatments of silk with additional quantity of ninhydrin as shown in Fig. 5. The total amino group content in silk should be the summation of the amino groups estimated in each treatment. Further, when we have adequate ninhydrin in the reaction medium there should be no residual amino group in treated silk. This was confirmed by treating 50 mg of silk with 16 ml of ninhydrin solution in a single reaction. The treated silk when further subjected to ninhydrin test showed only a negligible amount of residual amino groups. In an attempt to correlate the amino group content of silk obtained by the summation of the amino groups in successive treatments in the absence of inadequate ninhydrin concentration and the amino group estimated in single treatment using adequate ninhydrin, a close agreement was observed.

4 Conclusions

4.1 The optimum purple colour yield in silk-ninhydrin reaction was obtained in 35 min at 98°C, in 50 min at 90°C and in 80 min at 80°C.
4.2 The purple colour developed in silk-ninhydrin and glycine-ninhydrin reactions degraded on storage and totally disappeared in 48 h.
4.3 Experiments carried out with the inadequate quantity of ninhydrin showed the presence of residual amino groups in silk.
4.4 The need to use an adequate quantity of ninhydrin solution with respect to sample weight has been proved and a linear correlation has been obtained between the quantity of silk and the quantity of ninhydrin required.

References
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